ON THE SPECIFICITY AND RELATED PROPERTIES OF THE CRUSTACEAN CHROMATOPHOROTROPIC HORMONE

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Introduction

When the eye-stalks of several brachyuran crustaceans are removed, the animals become pale within two hours (1), (2), (3), (4). Injection of a sea-water extract of the extirpated eye-stalks into the blinded (eye-stalk-amputated) specimens results in the appearance of the dark coloration (3), (5). Although several kinds of chromatophores are involved in these color changes, the movements of the melanophores are chiefly responsible for the resulting external coloration, and in the following experiments were the only chromatophores studied in the fiddler crab, *Uca pugilator.*

Results

Method of Determining the Relative Activity of Eye-stalk Extracts

A new method of assaying the eye-stalk hormone was devised so that determinations could be made within an hour. The techniques of preparing the hormone, injecting the animals, and preparing the animals for the test were essentially the same as described previously (5). The plan of the method was as follows: Each of 8 groups of 15 blinded animals per group was injected with one of 8 different, known concentrations of hormone (1.0 E.S.[1]–0.005 E.S. per cc. of solution). The percentage of animals showing the slightest perceptible response (melanophore stellation) was determined at 5-minute intervals following the time of injection for a period of one hour. If the percentage of animals showing the slightest perceptible response at various concentrations is plotted against time, a series of steep, sigmoid curves is obtained whose inflection points intersect the ordinate at about 50 per cent. The times at which 50 per cent of the animals show the response at different concentrations range from 8 minutes for the strongest to 24 minutes for the weakest. A standard curve (Fig. 1) was then constructed, using a range of concentrations from 0.04

[1] The letters E.S. are the abbreviation for eye-stalk. The letters E.S.H. will be used as the abbreviation of eye-stalk hormone.

E.S./cc.–0.002 E.S./cc. Figure 1 can, therefore, be utilized for determining the relative strength of very dilute solutions of the hormone. For stronger concentrations (1.0 E.S./cc.–0.03 E.S./cc.) Fig. 2 was employed, indicating the relationship between concentration and percentage of animals showing the full response (complete melanophore expansion) at 1 hour following the time of injection. For still stronger solutions, the method previously described (5) was employed. By

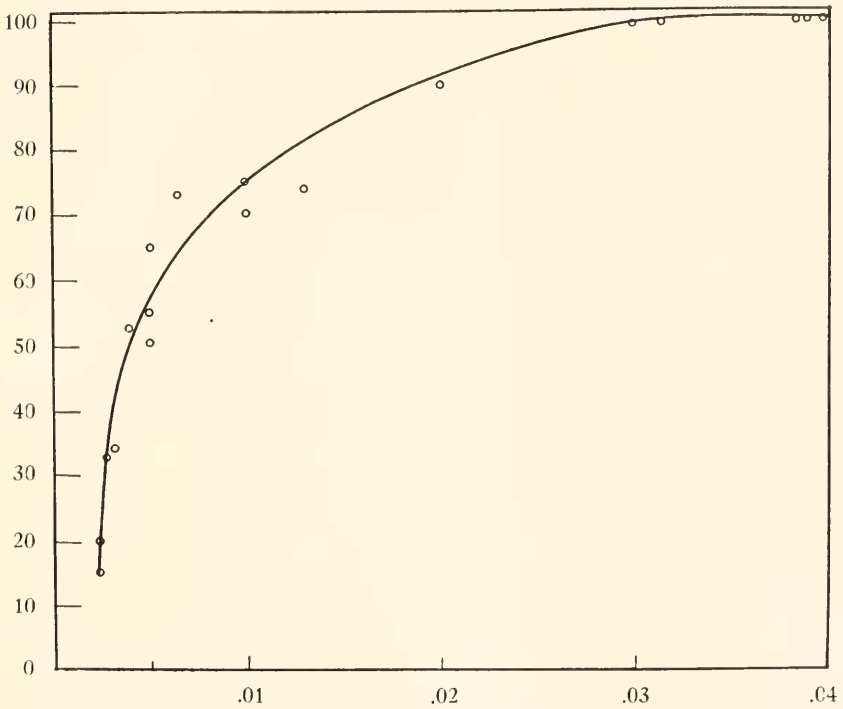

FIG. 1. Curve showing relationship between percentage of animals showing the slightest perceptible response (melanophore stellation) and concentration. Ordinate—percentage of animals responding; abscissa—concentration expressed as eye-stalks per cc. of solution.

using Figs. 1 and 2 as characteristic curves, the relative strength of an unknown concentration can be determined by taking a reading, at 30 minutes after injection, of the percentage of animals showing the slightest perceptible response, and another at 1 hour giving the percentage showing the full response.

A study of these and other curves not included in the text reveals

several points of interest concerning the nature of melanophore response in *Uca*. From the results shown by Fig. 1, where very small amounts of hormone were injected, it is evident that a certain threshold must be reached before the melanophores react and, moreover, that this threshold varies from animal to animal. With stronger doses, the

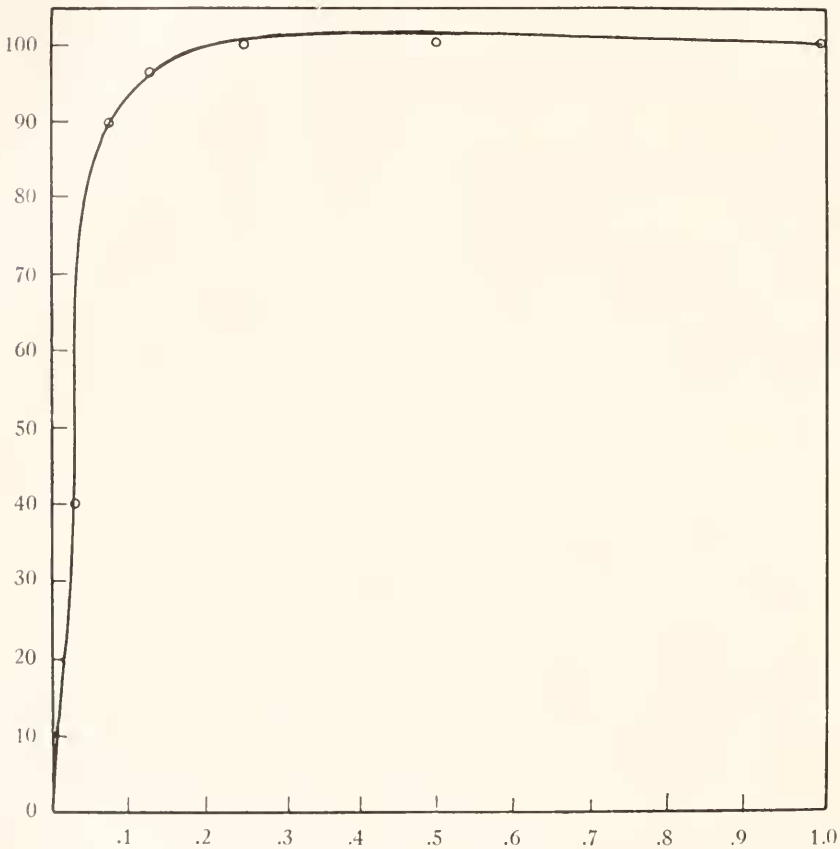

FIG. 2. Curve showing the relationship between percentage of animals showing the full response (complete melanophore expansion) and concentration of hormone. Ordinate—percentage of animals showing full response; abscissa—concentration expressed as eye-stalks per cc. of solution.

degree and the duration of melanophore expansion are exponentially proportional to the dosage injected. Thus, melanophores, like certain types of smooth muscle, show graded responses whose magnitude varies as the strength of the stimulus. For a complete expression among time, concentration of hormone, and the various phases of melano-

phore response, the construction of several nomograms is necessary. The curves already presented, however, suffice for the present studies.

Sensitivity of the Test; Estimation of the Amount of Hormone in the Eye-stalk

The strength of eye-stalk extracts, as read off from the curves, is not constant from animal to animal. Those from larger animals (5.0 grams) usually assay at higher values, although there is always some discrepancy in a group of the same size and sex. The sensitivity of the test may be illustrated by the following experiments: a sea-water extract of 1 eye-stalk (animal weight = 5.0 grams) was diluted until the total volume was 400 cc. Sixty-six per cent of the animals injected with 1/20 cc. of this solution responded. When further diluted to 600 cc., 50 per cent responded; to 800 cc., 33 per cent responded. With still greater dilutions, the percentage quickly fell to zero. We take the point at which 50 per cent of the animals respond as a reliable indication that an extract is active, and in the following discussion, we assume this point (50 per cent response) to indicate a minimal unit of activity, which is 1/600 of a *Uca* unit (5).

The dry or nearly dry weight of an eye-stalk is 2.0 mg. (average of 600 eye-stalks). From microscopic sections of the eye-stalks of *Uca pugilator*, we estimate the sinus gland of Hanström (6) to be, roughly, 1/100 that of the eye-stalk. Assuming the proportion of the active principle to be 1/100 that of the gland, which is probably a conservative surmise, the total amount of hormone in one eye-stalk is therefore about 0.2 γ. The sensitivity of this biological test is such that $0.2 \div 600 \times 20$ (since each animal is injected with 0.05 cc.) or 0.000016 γ of hormone can be detected. These figures, of course, are very rough but, we think, indicate the probable order of magnitude.

Specificity of Action

Thus far, extracts of only two other glands, the pituitary (7) and the subneural gland complex (8), have been found to produce melanophore expansion when injected into blinded *Uca*. We found accidentally that the injection of distilled water produces strong melanophore responses. In 55 crabs, blinded for from 2–8 days previously, the injection of distilled water produced in 15–20 minutes complete melanophore expansion in 90 per cent of the animals. After 1 hour, 50 per cent became pale, and after 2 hours, 90 per cent–100 per cent

became pale. Quantitatively, this effect is quite different from that produced by the eye-stalk hormone in any concentration. Injection of distilled water into 25 normal dark animals was without effect.

Since this reaction is of interest in relation to the general problem of hormone specificity, we examined several possibilities which might explain the effectiveness of distilled water. It is quite certain that injection of sea water does not produce any responses. Only 1 of 46 blinded animals injected with sea water responded weakly but positively, while no significant changes were detected in 10 normal dark animals also injected with sea water. Sea water, therefore, must be employed as the solvent when testing preparations of E.S.H. Since distilled water is acidic (pH 5.7) as compared with sea water (pH 7.7), we injected tap water (pH 7.0) into 10 blinded and 10 normal *Uca*, with results identical to those already described for distilled water. Identical results were also obtained with distilled water made alkaline (pH 8.0) by the addition of 0.01 N NaOH. The acidity of distilled water is therefore not responsible for its chromatophorotropic activity.[2]

Another difference between the action of the hormone and that of distilled water was obtained by injecting various dilutions of sea water with distilled water. The following proportions of sea and distilled water were tried:

Sea Water		Distilled Water	Chromatophorotropic Activity
1 part	plus	1 part	Inactive
1 part	plus	2 parts	60 per cent of animals respond
1 part	plus	3 parts	80 per cent of animals respond
1 part	plus	6 parts	80–100 per cent of animals respond
1 part	plus	10 parts	80–100 per cent of animals respond
1 part	plus	16 parts	80–100 per cent of animals respond

Since the injection of 0.05 cc. distilled water must dilute the blood of a crab appreciably, osmotic effects were studied. Assuming the freezing point depression of *Uca* blood to be close to that of lobster blood, we injected sucrose (1.3 M) isosmotic with the latter into 20 blinded crabs. No melanophore expansion resulted. Sucrose (0.95 M) isosmotic with sea water, however, produced a slight reaction in 20 per cent of the animals. It seemed, therefore, that the effect of distilled water may be due to the resulting hypotonic condition of the blood. The following salts isosmotic with sea water were also tested:

[2] The pH of distilled, tap, and sea water at Woods Hole was determined by means of the glass electrode.

Salt		Blinded Specimens (20 animals per test) General Effects	Chromatophorotropic Activity (per cent of animals responding)
NaCl	0.52 M	—	30
$MgCl_2$	0.51 M	—	20
$CaCl_2$	0.34 M	—	20
KCl	0.53 M	Muscular twitchings and death	0
Na_2HSO_4	0.4 M	Immediate prostration, death after 1 hour	10
LiCl	0.52 M	Immediate twitchings of legs	20
200 per cent sea water		—	0

While the effect of drugs on vertebrate melanophores has been determined, no similar investigation has been made on crustacean melanophores. Sixteen different, chemically-pure drugs were injected into both normal and blinded crabs. One hundred γ of the drugs, dissolved in sea water, was injected into each of 10–20 animals of both groups. Several drugs produced violent, convulsive movements, prostration or twitchings of the legs while others were entirely without muscular effects. Table I summarizes the action of 100 γ of the drugs:

TABLE I

Effects of sixteen drugs on normal and blinded crabs

Drug	Blinded (Pale Animals)		Normal (Dark Animals)	
	General Effect	Chromatophorotropic Activity (Melanophore expansion)	General Effect	Chromatophorotropic Activity (Melanophore contraction)
Atropine sulphate	—	—	—	—
Morphine sulphate	—	—	—	—
Acetyl choline	—	20% (slight)	Shedding of legs	—
Histamine	Paralysis of legs	—	Slight paralysis	—
Eserine sulphate	Prostration and death	—	Not performed	
Pilocarpine HCl	—	—	—	—
Cocaine HCl	Rigidity	30% (slight)	Rigidity	—
Brucine sulphate	Paralysis	—	Sluggishness	—
Veratrine sulphate	Instantaneous death	—	Instantaneous death	50% slightly pale
Curare	Paralysis	50% (slight)	Paralysis	—
Strychnine SO_4	Temporary paralysis	20% (slight)	Paralysis	—
Guanidine	Prostration	20% (slight)	—	—
Chlorbutanol	—	20% (slight)	—	—
Caffein	—	20% (slight)	—	—
Nicotine	Prostration	40% (slight)	Prostration	—
Hyoscine HBr	—	80% positive	—	—

None of these drugs with the exception of hyoscine hydrobromide produced definite, positive results in blinded specimens, and all were without effect on the melanophores in normal dark specimens. These drugs (with the one exception) have therefore no direct or indirect action on contracted or expanded melanophores. We did not repeat these injections in normal animals during the night (nocturnal or pale phase) although possibly some of these drugs may effect a release of the hormone from the sinus gland, which appears to be under nervous control (5).

Adrenalin, practically universally, produces melanophore contraction in vertebrates in extremely small doses. Adrenalin, in various dilutions from 1 : 1,000 to 1 : 10,000 (dilutions greater than 1 : 1,000 being made with sea water) was without significant melanophore responses when injected into either normal or blinded specimens. Strong doses usually produced death in normal animals, while 20 per cent of the blinded animals showed slight melanophore stellation. The activity of adrenalin was confirmed by injecting 0.2 cc. of each dilution subcutaneously in normal dark catfishes. This experiment illustrates effectively the point made elsewhere (4) that melanophores of various animals do not always react in the same way to the same substance, for example, chemically pure adrenalin hydrochloride. It is only logical to think of a response to a hormone in terms of the interaction between the responding tissue and the hormone in question, not solely in terms of the hormone itself. If the same type of responding tissue in two different animals is physiologically and anatomically different, as are the melanophores of crustaceans and vertebrates, it is not surprising that they react in different ways to the same substance. The ineffectiveness of adrenalin in contracting crab melanophores is therefore not a puzzling phenomenon. In fact, it is almost surprising that in one case (7) (4), intermedin produces the same response in crab and vertebrate melanophores.

Do Organs Other than the Eye-stalk Produce the Hormone?

This question has been discussed in detail (5) but as yet no conclusive answer to it has been given. It has been reported (9) that ventral nerve cord extracts of *Palæmonetes* are slightly active on the chromatophores of blinded *Palæmonetes*, and therefore that the ventral nerve cord produces the active principle or principles. A criticism of this conclusion is the possibility that the extracts, prepared from normal animals, may have contained traces of E.S.H. present in the blood bathing the nerve cord.

We have extracted several organs of normal pale, normal dark,

and blinded crabs (blinded previously for various periods of time), and tested the extracts on crabs blinded previously for from 2–8 days. For purposes of comparing the potency of any organ found to be active with that of the eye-stalk, the minimal unit of activity as already described was employed. The wet weight of an eye-stalk is about 6.0 mg., and consequently 600 cc. ÷ 6 mg. or a 0.001 per cent solution of the eye-stalk represents a minimal unit. Stomach, liver, muscle and heart tissue of both normal dark and blinded specimens were extracted by the usual method and prepared in a 0.1 per cent solution; in other words, 100 times more concentrated than that representing a minimal unit of eye-stalk extract. All extracts were found to be completely inactive. Finally, we resorted to extraction of entire, normal dark crabs, immediately after extirpating the eye-stalks. The extract was prepared in a 2 per cent solution, or 2,000 times more concentrated than the minimal unit of eye-stalk extract. When tested, it was found to be 5 times stronger than the minimal unit. Repetition of this experiment with normal pale animals and animals blinded for from 2 days to 1 month gave identical results. We must conclude that either the hormone is present or being produced by some tissue in the body even in the absence of the eye-stalks, or that the positive result is an artifact brought about by the injection of a hypertonic solution. If the former is true, it can be of little or no significance in the normal chromatic physiology of these animals, as shown subsequently.

Behavior of Isolated Leg Melanophores

The behavior of isolated scale melanophores of fish to various ions and organic substances has been studied (10) for a long time. One of the most remarkable results is the behavior of *Fundulus* scale melanophores to sodium and potassium ions, expanding to the former and contracting to the latter. We have studied the melanophores of crabs to various substances by cutting off the legs with a fine scissors at a point near the articulation of the femur with the body, and immersing these isolated legs into various solutions. The results are summarized in Table II, readings being taken every fifteen minutes after immersion in the solutions and continued for 5 hours.

The results are not particularly illuminating. It is significant, however, that the effect of distilled and tap water is similar to that produced when they are injected into the animal. Sea water also acts in the same fashion as when injected into pale animals. The fact that dark legs paled in sea water after 1½ hours may be due to the diffusion, destruction, or inactivation of E.S.H. already present in

the legs. This resembles the last phase of a typical response of blinded crabs to the injection of E.S.H., for when the melanophores begin to contract it requires about 1½ hours to attain complete pallor (5). In view of this, and the slowness of melanophore reaction in crabs, we cannot speak of "ionic effects" on melanophores provided the responses are produced well within an hour. Thus, lithium, sodium, potassium, and calcium chlorides all produce a rapid contraction of expanded melanophores, and consequently, there is no difference, as

TABLE II

Legs of blinded crabs (melanophores contracted)

Solutions		Results of Immersion
$MgCl_2$	.37 M	No change for 4 hours, then expansion of femur melanophores
Sucrose	1.30 M	Same as above, although erythrophores expand within 30 minutes
LiCl	.52 M	No change
Na_2HPO_4	.40 M	Expansion within 15 minutes
NaCl	.52 M	Slight stellation for first hour, then contraction
$CaCl_2$	.34 M	No change for 4 hours, slight expansion on fifth hour
KCl	.53 M	Slight stellation for 1½ hours, then contraction
Sea water		No change during 5 hours
Tap water		Expansion within hour, lasting for 5 hours on femur
Distilled		Same as above

Legs of normal dark crabs (melanophores expanded)

Solutions		Results of Immersion
$MgCl_2$	.37 M	Slight contraction in 15 minutes to 4 hours, then expansion on femur
Sucrose	1.30 M	Slight contraction in ½ hour, expansion after 2 hours
LiCl	.52 M	Contraction in 15 minutes to 5 hours
Na_2HPO_4	.40 M	Remain expanded for 5 hours
NaCl	.52 M	Contraction in 15 minutes to 5 hours
$CaCl_2$	.34 M	Contraction 15 minutes to 4 hours, then expansion on femur
KCl	.53 M	Contraction 15 minutes to 5 hours
Sea water		Contraction after 1½ hours
Tap water		Slight contraction after 1½ hours, but expansion after 4 hours
Distilled		Same as above

found in fish melanophores, between the effects of NaCl and KCl. Melanophores in the legs of blinded animals remain more or less contracted in these solutions for 4 hours, so that the effect of these various cations is the same regardless of the original state of the melanophores. However, since the chloride ion is the common anion to all four salts, the result may well be due to it. We have not pursued this aspect further for it is outside the scope of this paper. Isolated leg melanophores of crabs are not valuable material for such an investigation. Their responses are slow, and usually irregular. Most frequently the

reaction starts at the cut surface of the femur and progresses inwardly towards the tibia. Occasionally, the melanophores of the entire femur respond simultaneously, and rarely do the pigment masses of the tibia react prior to those of the femur.

Is the Pale State Constant in Blinded Animals?

It was stated previously that if tissues other than the eye-stalk are capable of producing E.S.H. they must play an insignificant part, if any, in the normal chromatic physiology of the animals. The basis for this belief is that animals once blinded remain in a pale state as long as they remain alive. In all of the experiments, 15–20 blinded animals were maintained in large crystallizing dishes containing about ½ inch of sea water which was changed daily. Under these conditions, blinded animals have been maintained for 2 months, during which they remained entirely pale.

When blinded animals die, they turn quite dark. Even previous to death, the animals turn gradually dark, and remain dark for some time after death. After observing several hundred blinded animals from day to day, we can state that pallor after enucleation of the eye-stalks is largely constant so long as the crabs remain in a healthy state. Thus far, we have not observed regeneration of the eye-stalk, although several puzzling observations, exceptions to the above statement, have suggested this as a possible explanation. The following protocol illustrates the condition of the melanophores in blinded animals:

Aug. 31—158 animals blinded
Sept. 1—100% pale
Sept. 2—98% pale; 2% dark (dead)
Sept. 4—1% slightly dark; 99% pale
Sept. 5—during day 100% pale; during night 100% pale
Sept. 6–13—animals divided into 4 groups:
white background during day —100% pale
white background during night—100% pale
black background during day —100% pale
black background during night—100% pale
Sept. 13—142 animals remaining:
3% slightly dark
5% fully dark } = A
92% pale = B
Sept. 14—A = 45% dead; 55% dark; B = 93% pale
Sept. 15—A = 33% dark; 66% pale (day)
100% pale (night)
Sept. 16—A = 84% dark; 16% pale (day)
84% pale; 16% dark (night)

The protocol illustrates that over 90 per cent of the animals blinded for 2 weeks remain continuously pale. From 2 per cent–4 per cent, at the end of two weeks, show a slight diurnal rhythm. Further proof of this behavior was obtained by placing 40 animals, blinded two weeks previously, in a glass dish near a window and 25 similar animals in a glass dish in total darkness.

	Exposed to Normal Day and Night Conditions	Darkness
Sept. 14 P.M.	95% P; 5% slightly D	100% P
Sept. 15 A.M.	95% P; 5% slightly D	100% P
Sept. 15 P.M.	95% P; 5% slightly D	100% P
Sept. 16 A.M.	70% P; 30% slightly D	100% P
Sept. 16 P.M.	97% P; 3% slightly D	100% P
Sept. 17 A.M.	67% P; 33% slightly D	100% P

P = pale. D = dark.

A certain percentage of blinded animals is therefore capable of undergoing periodic changes in color. Usually the extent of melanophore expansion is only slight stellation, yet this is a positive reaction. On examination, it was noticed that the stubs of the eye-stalks in those animals showing diurnal rhythm had healed, and were somewhat elongated. However, the stubs were scraped, cut off, or pulled out of their sockets without altering the slightly dark coloration, and consequently the possibility that the regeneration of the sinus gland had taken place was eliminated. That the slight darkening observed in blinded animals during daylight may be due to the direct action of light is improbable because this phenomenon is limited to only a few animals and occurs only after some time has elapsed following extirpation.

Immersion of Animals in Distilled Water

Fifteen blinded animals were placed in a large dish containing distilled water to a depth of one inch, which was changed daily. After 2 days of this treatment, 9 of the animals became dark (ranging from intermediate to complete darkness) while 6 remained pale. These were segregated, and listed as *A* and *B* groups respectively. On the third day, 4 of the animals in *A* were placed in sea water and designated as *C*, and the 3 groups watched daily:

	A (5 animals)	*B* (6 animals)	*C* (4 animals)
4th day	All dark	1 pale, 5 dark	All pale
5th day	4 dark 1 pale	1 pale, 5 dark	All pale
6th day	3 dark 2 pale	2 pale, 4 dark	All pale

At the end of the week, one-third of all the animals had died, the remaining ten were placed in one dish, containing distilled water. On the eighth day, 6 had become pale, and on the ninth day all had become pale, remaining in this state for 4 more days when all but 2 died. One was now injected with distilled water, which promptly evoked an expansion within 15 minutes, but this response vanished within the next 15 minutes. The other was injected with 0.1 E.S. and responded in 15 minutes, remaining dark for 2 hours.

Distilled water has then the same melanophoric effect when animals are immersed in it as when it is injected into the body spaces. After a week, however, the animals become refractory and the dark coloration is gradually lost, but the animals have not lost the ability to respond to injections of E.S.H. or distilled water.

Chemical Characteristics of E.S.H.

The properties of E.S.H. reported earlier (5) have been confirmed and extended. However, in our experiments, the solubility of E.S.H. in various solvents was determined after the following preliminary treatment: 100 eye-stalks were extracted three times with 10 cc. of distilled water by boiling. The solution was filtered and the filtrate reduced to a volume of 10 cc. under a warm current of air. Two volumes of either pyridine, ethanol or acetone were added, and the solution cooled to 5° C. for one day. A heavy red precipitate forms which is discarded after centrifugation. The supernatant fluid contains practically all of the activity, and after drying, was used as stock material. One extraction with 10 cc. of the following solvents was made of the dry powder equivalent to 10 E.S., and both soluble and insoluble fractions assayed:

Solvent	Percentage of Activity Soluble
	per cent
Absolute ethyl alcohol	45
Absolute methyl alcohol	60
95% ethyl alcohol	60
Absolute acetone	0
Ethyl ether	0
Petroleum ether	1
Benzene	1
Chloroform	0
Ethyl acetate	0
Pyridine	6
95% acetone	2
90% acetone	20
95% methyl alcohol	80

Due to the small amount of material available, we have not concentrated on the purification of the hormone. However, if the stock

material is repeatedly extracted with 95 per cent methyl alcohol, most of the activity can be collected as a soluble fraction, which, on basis of activity per milligram of dry weight, represents a tenfold purification.[3] Aqueous acetone seems to destroy some of the hormone for we have not been able to account for all of the original activity present before fractionation by totalling the activity of the soluble and insoluble fractions.

The hormone is apparently soluble in water throughout the pH range. Stock material (20 E.S.) was easily dissolved in 10 cc. of 0.1 N NaOH as well as in 10 cc. of 0.1 N HCl with the formation of a flocculent, inactive precipitate. If 10 E.S. are dissolved in 10 cc. of 0.1

TABLE III

Diurnal rhythm of *Uca pugilator*

Time of Observation	Normal Day and Night Conditions				Constant Darkness	
	Normal Animals			Animals Blinded 2 Weeks Previously *	Normal Animals	Blinded 2 Weeks Previously
					Indeterminate Background	
	White Background 40 Animals	Black Background 40 Animals	Indeterminate Background 40 Animals	Indeterminate Background 40 Animals	25 Animals	25 Animals
September 13 11:00 A.M.	100% D	100% D	100% D	75% I-P	100% D	4% D
7:30 P.M.	82% P	100% D	100% D	100% P	—	—
12:45 A.M.	100% P	50% I; 50% P	80% P	100% P	50% P; 50% I	100% P
September 14 10:00 A.M.	100% D	100% D	100% P	70% I-P	100% D	100% P
8:00 P.M.	26% P; 74% I	12% P; 88% P-I	80% P	98% P	60% P	100% P
10:00 P.M.	80% P; 20% I	18% P; 82% I-D	100% P	98% P	80% P	100% P
September 15 10:00 A.M.	100% D	100% D	100% D	50% I-D	100% D	100% P
4:30 P.M.	100% D	98% D	100% D	87% P	25% P	100% P
10:30 P.M.	90% P	50% P	66% P	87% P	92% P	100% P
September 16 11:15 A.M.	100% D	100% D	100% D	40% I	96% D	100% P
10:30 P.M.	100% P	25% P	100% P	90% P	81% P	100% P
September 17 10:30 A.M.	100% D	100% D	100% D	40% I	81% D	100% P

[3] It was reported (8) that the hormone was precipitated from 95 per cent acetone by the addition of ether. This is an error since in this case the hormone was precipitated from 95 per cent methanol, not 95 per cent acetone.

TABLE III—*Continued*

Time of Observation	All Animals Now Exposed to Constant Light (75 Watt Electric Bulb)					
	Normal Animals			Animals Blinded 2 Weeks Previously *	Normal Animals	Blinded 2 Weeks Previously
					Indeterminate Background	
	White Background 40 Animals	Black Background 40 Animals	Indeterminate Background 40 Animals	Indeterminate Background 40 Animals	25 Animals	25 Animals
September 18 11:15 A.M.	100% D	100% D	100% D	40% I	100% D	55% I-P
3:00 P.M.	98% D	100% D	100% D	25% I	100% D	50% I-P
10:15 P.M.	40% P; 60% I-D	14% P	33% P	3% I; 97% P	33% P	100% P
September 19 12:30 P.M.	100% D	100% D	100% D	25% I	100% D	25% I-P
10:00 P.M.	30% P	20% P	50% P	92% P	50% P	100% P
September 20 12:30 P.M.	100% D	100% D	100% D	12% I	100% D	25% I-P
11:00 P.M.	75% P	33% P	75% P	92% P	50% P	75% P
September 21 12:30 P.M.	100% D	100% D	100% D	14% I	100% D	50% I
10:30 P.M.	50% P	20% P	50% P	90% P	33% P	90% P
September 22 12:40 P.M.	100% D	100% D	100% D	16% I	100% D	42% I
10:20 P.M.	50% P	16% P	60% P	94% P	50% P	90% P
September 23 11:10 P.M.	50% P	6% P	33% P	91% P	33% P	100% P
September 25 1:00 A.M.	50% P	14% P	80% P	96% P	12% P	75% P

P = pale.
D = dark.
I = intermediate.
I–D = intermediate to dark.
I–P = intermediate to pale.

* These were chosen from several hundred specimens because they were not entirely pale.

NaOH and, after 3 minutes, neutralized to pH 7.0 and tested, complete activity is found. If a similar solution is placed at 100° C. for ten minutes, total loss of activity occurs. Ten E.S. in 10 cc. of 0.1 N HCl placed at 100° C. for ½ hour, neutralized, and tested, retains its original activity. In fact, HCl seems to potentiate the hormone somewhat, although we have not studied this carefully. Attempts to regenerate the activity of stock material treated for 15 minutes with NaOH by boiling it with an equivalent amount of HCl after neutralization were unsuccessful.

The Diurnal Rhythm of Uca pugilator

It has been stated (5) that the diurnal chromatic rhythm of *Uca pugilator* proceeded under constant illumination or darkness, and regardless of background. This statement was based upon observations made on only the melanophores of the abdominal segments, since the ventral surface of these crabs can be most easily observed. Since all the experiments reported here deal with the reactions of leg melanophores, we studied for 2 weeks the behavior of the leg melanophores during the periodic change in coloration. Essentially, the same situation as previously described was found, but several new facts of interest were discovered. The following protocol summarizes the observations upon 170 specimens kept under various conditions:

It will be seen that when normal animals are exposed to normal day and night environment, the diurnal rhythm proceeds as described previously on both white and indeterminate backgrounds. On black background, however, the appearance of the nocturnal hue is delayed and does not occur in a certain percentage of animals. Under constant light, the same situation is true except that fewer animals on all backgrounds show the nocturnal phase. Animals maintained in darkness show the nocturnal coloration at night but when exposed to constant light, the number of specimens becoming pale at night is decreased. Two new facts therefore emerge: (1) that black background delays and inhibits the pale phase more effectively under conditions of constant illumination than under normal condition daylight and darkness, (2) that constant light does the same regardless of background.

Observations on the ventral surface of these specimens were made simultaneously but excluded from the protocol. If readings were based upon the behavior of the abdominal melanophores alone, the protocol would indicate that the pale phase occurred in practically all of the specimens regardless of background or of conditions of illumination. There is, therefore, a decided difference between the reactions of the leg and abdominal melanophores.

The diurnal rhythm of *Uca* has been explained on the assumptions that during the day, E.S.H. is released into the blood stream and that during night, release is stopped. On this basis, it must be assumed that the abdominal melanophores are more sensitive than those of the legs to the disappearance of the hormone from the blood stream. In addition to the circumstantial evidence obtained by watching single animals or groups of animals during the diurnal rhythm, further proof was obtained by removing the eye-stalks of 15 dark animals during the day. After 1 hour, 20 per cent of the animals showed white

abdomens but the legs remained dark in all cases. After 1½ hours, 66 per cent of the specimens showed white abdomens and 20 per cent, pale legs. After 2½ hours, all showed pale abdomens while 70 per cent showed pale legs. This experiment, therefore, indicates quite clearly that the abdominal melanophores respond more rapidly than leg melanophores to the loss of E.S.H. from the blood, as brought about by removal of the eye-stalks.

The assumption that the pale phase of *Uca* is due to the absence of E.S.H. from the circulation during the night is based on no direct evidence inasmuch as we have not succeeded in obtaining the blood of *Uca* for assaying E.S.H. The evidence for this belief is, therefore, indirect, and is based on one fact—that pallor in *Uca* is always associated with and due to the loss of the eye-stalks, and hence removal of the chief if not the only significant source of E.S.H. However, it may be possible that the melanophores become refractory during the night to the eye-stalk hormone (which may be thought of as being secreted constantly) thus ushering in the pale phase, or that the pale phase is due to a melanophore-contracting hormone, concentrating nerves, or some unknown factor. The first possibility was negated by injecting some E.S.H. into 20 normal animals showing the nocturnal phase. All responded normally by darkening. The second possibility has no experimental basis inasmuch as no extract of *Uca* has been prepared which induces pallor, and as chromatophore nerves have not been demonstrated in the Crustacea.

Consequently, the explanation of the diurnal rhythm of *Uca* on the basis of the presence and absence in the blood stream of one hormone (E.S.H.) seems to be the most economical, and in conflict with no observational and experimental facts. The effect of constant light and of black background can therefore be thought of as prolonging the release of the eye-stalk hormone into the circulation, or as inhibiting or delaying the process by which the release of the hormone from the gland is stopped.

Discussion

Since most of the data have already been analyzed, we would limit this to a short theoretical discussion on the nature of action of the eye-stalk hormone. It is evident that the problem of the endocrine control of chromatophores in crustaceans is not as simple as has been regarded. While in general the comparative endocrinology of melanophore responses in both vertebrates and invertebrates is rather well understood, we have no inkling of the microphysiology of either the melanophores or the chromatophorotropic hormones in action. In

fact, there is nothing known, with one or two exceptions, concerning the intimate mechanism by which a hormone is able to affect a tissue. The finding that distilled water expands the melanophores of blinded crabs may furnish a clue as to the manner in which the eye-stalk hormone acts, even though the reactions may not be comparable. Since the characteristic action of E.S.H. is the induction of protoplasmic movements in the melanophores, one is inclined to suspect the ionic or osmotic environment as the regulator of melanophore movements. This may or may not be reasonable but, at least, the effect of distilled water can be attributed to the resulting hypotonicity of the blood which in turn might cause a rapid shift in the osmotic equilibrium between the blood and tissues. The expansion of the melanophores might then result from such a change in the osmotic or ionic environment.

There are two possibilities concerning the way in which E.S.H. acts on the melanophores—directly or indirectly. Discussing the second possibility first, it is quite possible that the eye-stalk hormone may not have any relation to changeable coloration at all, but that it regulates the salt or water balance of the animal. The effect on melanophores may well be indirect, resulting from osmotic or ionic changes between the blood and tissues when the hormone is normally present and absent in the blood stream. This would mean, of course, that the chromatophorotropic hormone is only secondarily chromatophorotropic; its main function being in regulating some other process. Indeed, it has been maintained (11) that since this hormone is found in crustaceans which do not show color changes or even contain chromatophores, it must perform some function more important than that of regulating color changes. Such an argument is helpful for the above discussion, but we feel that the presence of a particular hormone may not necessarily mean that it must be performing some function. For example, intermedin is abundantly present in the pituitary glands of both birds and mammals, yet its presence is not correlated with any functional significance. Similarly, the presence of estrogenic hormones in the mammalian testes does not seem to be correlated with any of the normal processes in the reproductive physiology of the male. There are other instances of this kind, but of course some function for the hormones mentioned may be discovered eventually.

To the effect that E.S.H. acts directly on the melanophores, we might add that all of our curves relating response to concentration and time are of a type which has been assumed to represent reversible compound formation on cell surfaces—but this is all that we can marshal in support of a direct action. Isolated leg melanophores will expand when immersed into a solution of sea water containing E.S.H.,

but this is of course no indication of a direct action. Similarly, the effectiveness of various salts on isolated melanophores offers nothing to indicate that the action of E.S.H. is direct or indirect. The determination of the mechanism of action of the eye-stalk hormone belongs properly to general physiology and is perhaps one of the most significant aspects of melanophore physiology. It is hoped that some of these observations will furnish a basis for further investigation.

In concluding, it can be mentioned that a search for other endocrine functions of the eye-stalk such as have already been found (regulation of heart (12), calcium metabolism (13)) will be of importance in establishing the proper rôle which the eye-stalks play in regulating the pigmentary effectors. The hormone is not indispensible to the life of crustaceans. Blinded crabs molt frequently (perhaps more frequently than normal specimens according to our impression) and if well fed can be maintained in a healthy state for several months, and perhaps indefinitely.

Summary

1. A method for determining within an hour the relative activity of eye-stalk extracts of *Uca pugilator* has been described.

2. The amount of hormone present in one eye-stalk of *Uca* (5.0 grams) was calculated to be 0.2 γ. The minimal amount of hormone detected by the method described was calculated to be 0.000016 γ, which represents a minimal unit of eye-stalk activity.

3. Distilled water is effective in expanding melanophores when injected into blinded specimens. The effect seems to be related to osmotic changes induced in the animal. Excepting the effect of distilled and tap water, the response of blinded animals to the eye-stalk hormone seems to be quite specific. Sixteen different drugs were ineffective in causing melanophore expansion.

4. Blinded animals immersed in distilled water become and remain dark for a week, after which they become refractory. However, they have not lost the ability to respond to injections of either eye-stalk hormone or distilled water.

5. Extracts of several organs other than the eye-stalk were found to be inactive on blinded specimens, even when 100 times more concentrated than a minimal unit of eye-stalk extract. Extracts of entire bodies, 2,000 times more concentrated than a minimal unit, were active.

6. Isolated leg melanophores respond slowly and irregularly to various ions. Na, K, Ca, Li chlorides all induce melanophore contraction. Na and K do not produce opposed responses in isolated leg melanophores as they do in isolated scale melanophores.

7. The relative solubility of the eye-stalk hormone in various organic solvents was determined. The hormone is inactivated by alkali, but the activity cannot be regenerated by treatment with acid.

8. Animals blinded for 2 weeks or more remain continuously pale, with the exception of 2–4 per cent of the specimens, which show slight periodic changes in coloration.

9. The diurnal rhythm of *Uca* is affected by constant light, and black background, both of which delay the appearance of the nocturnal hue on the legs.

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